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Single-Chain Fv-Based Anti-HIV Proteins: Potential and Limitations

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The existence of very potent, broadly neutralizing antibodies against human immunodeficiency virus type 1 (HIV-1) offers the potential for prophylaxis against HIV-1 infection by passive immunization or gene therapy. Both routes permit the delivery of modified forms of IgGs. Smaller reagents are favored when considering ease of tissue penetration and the limited capacities of gene therapy vectors. Immunoadhesin (single-chain fragment variable [scFv]-Fc) forms of IgGs are one class of relatively small reagent that has been explored for delivery by adeno-associated virus. Here we investigated the neutralization potencies of immunoadhesins compared to those of their parent IgGs. For the antibodies VRC01, PG9, and PG16, the immunoadhesins showed modestly reduced potencies, likely reflecting reduced affinities compared to those of the parent IgG, and the VRC01 immunoadhesin formed dimers and multimers with reduced neutralization potencies. Although scFv forms of neutralizing antibodies may exhibit affinity reductions, they provide a means of building reagents with multiple activities. Attachment of the VRC01 scFv to PG16 IgG yielded a bispecific reagent whose neutralization activity combined activities from both parent antibodies. Although the neutralization activity due to each component was partially reduced, the combined reagent is attractive since fewer strains escaped neutralization.

Developing an effective human immunodeficiency virus type 1 (HIV-1) vaccine has been a great challenge for more than 25 years. Results from the RV144 vaccine trial in Thailand suggested that a partial degree of protection from infection was achieved (32), but whether and how a more effective vaccine can be developed remain open questions (39, 40). Difficulties in making an effective vaccine result in part from the humoral immune response against HIV-1, in which the antibodies produced are generally strain specific and can be quickly evaded by the rapidly mutating virus (43). Highly potent cross-strain anti-HIV antibodies have been isolated (5, 35, 41, 42, 45), but the unresolved problem is how to elicit these rare antibodies.

Although neutralizing antibodies have shown limited efficacy for controlling an established HIV-1 infection (26, 31, 38), the observation that most new infections appear to be initiated by only one or a few viral particles (17, 33, 34) highlights the potential for antibodies to provide sterilizing immunity. Passive immunization studies with broadly neutralizing antibodies have demonstrated their ability to protect animals from an HIV/simian immunodeficiency virus (SIV) chimera challenge (2, 11–13, 23, 24, 29, 30, 36). Hence, an alternative approach to prophylaxis is to deliver the genes for potent anti-HIV proteins to provide long-lasting protection. A successful demonstration of this approach in rhesus macaques using adeno-associated virus (AAV) as the gene delivery vehicle has been achieved (15). AAV is an attractive vector due to its long-term gene expression and low toxicity (10). However, the use of AAV vectors imposes a size restriction on the gene delivered: expression from AAV vectors with genomes larger than 4,900 bases is greatly attenuated (7). This can make it difficult to use AAV for delivery of large proteins, such as IgG antibodies, which include a heavy chain (HC) with four domains (the Fab heavy (V_H) and constant heavy 1 (C_H1) domains and the Fc C_H2 and C_H3 domains) and a light chain (LC) with two domains (the Fab variable light (V_L) and constant light (C_L) domains) (Fig. 1). Self-complementary AAV vectors are one means of achieving high expression levels (25); however, size restrictions for these vectors prevent simultaneous incorporation of conventional antibody heavy and light chain genes.

To achieve high transduction levels, a smaller immunoglobulin architecture was used in the AAV-mediated gene therapy experiments in rhesus macaques (15): single-chain fragment variable (scFv) units attached to an Fc domain (an scFv immunoadhesin comprising IgG V_H, V_L, C_H2, and C_H3 domains, here referred to as an immunoadhesin or IA) (Fig. 1). A wide variety of Fc fusions have been developed over the last 20 years to take advantage of this architecture's key benefits: avidity provided by homodimeric Fc, serum persistence provided by the Fc region due to FcRn-mediated protection from catabolism, and a size large enough to avoid filtration by the kidneys (14). An scFv unit, in which V_H is fused to V_L with a short linking region usually composed of glycines and serines, generally retains the antigen binding functionality of its parent Fab, although scFv is only about 1/2 the size of an intact Fab. Although scFv-based reagents have been under development for many years, their overall potential as drugs remains uncertain (28).

Several broadly neutralizing and highly potent antibodies against HIV-1 have recently been isolated from infected individuals. Two such antibodies, PG9 and PG16, target a quaternary epitope involving the V1-V2 and V3 variable loops of gp120 (42). Another class of antibodies, typified by antibody VRC01, targets the CD4-binding site of gp120 (44). The efficacy of gene therapy reagents derived from these antibodies depends on a number of factors, including their potency, strain coverage, *in vivo* stability, effector function, and serum concentrations that can be achieved.

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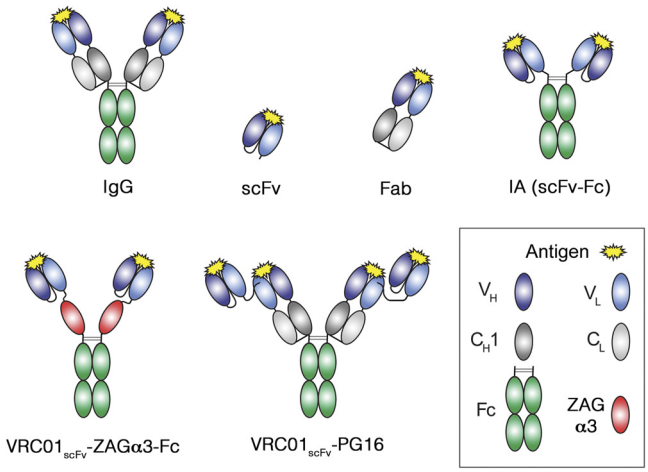

FIG 1 Schematic depiction of antibody reagent architectures. V_H, variable domain of the IgG heavy chain (HC); V_L, variable domain of the IgG light chain (LC); C_H1, constant region 1 of the HC; C_L, constant region of the LC; Fc, C_H2 and C_H3 domains of dimerized HCs; scFv, single-chain fragment variable (V_H and V_L domains of an IgG). The scFv shown is V_H followed by V_L; scFvs can also be constructed as V_L followed by V_H.

To help inform decisions relating to the architecture of potential reagents, we systematically compared the potencies of IAs with their IgG and Fab counterparts. We also explored the potential to combine VCR01 and PG9/PG16 activities to produce a single reagent with two gp120 specificities.

MATERIALS AND METHODS

Materials. Sequences for all constructs are in Fig. S1 in the supplemental material. VRC01 IgG was expressed using plasmids VRC8551 and VRC8552, provided by Gary Nabel (Vaccine Research Center, NIH). VRC01 Fab was expressed using a truncated VRC8552 heavy chain gene sequence encoding a 7×-His tag and stop codon after the C_H1 domain. VRC01 IgG-2A was expressed using plasmid VRC9715, which contains a picornavirus 2A peptide sequence (37) between the heavy and light chain genes. VRC01 IA was expressed using plasmid VRC9713 (provided by Gary Nabel), which encodes an IA protein in which a VRC01 scFv [V_H domain connected via a $(Gly_3Ser)_4$ linker to the V_L domain] is fused to the Fc region from human IgG1. A VRC01 scFv gene was constructed by truncating the VRC01 IA gene by inserting a 6×-His tag and stop codon after the V_L domain.

Genes encoding the variable regions (V_H and V_L) or the intact light chain (V_L-C_L) of PG9 and PG16 antibodies (Abs) were synthesized (BlueHeron Biotechnologies or Integrated DNA Technologies) based on sequences provided by Dennis Burton (The Scripps Research Institute). Intact IgG genes were constructed by subcloning the relevant variable sequences onto a human IgG1 sequence. The designs of the PG9 and PG16 IAs were patterned after the rhesus IAs described previously (15); thus, PG9 IA was constructed as V_H-$(Gly_4Ser)_3$-V_L-Fc, and PG16 IAs were constructed as V_L-$(Gly_4Ser)_3$-V_H-Fc and V_H-$(Gly_4Ser)_3$-V_L-Fc; for these IAs, the Fc sequence was that of human IgG2. PG9 and PG16 Fabs were expressed using truncated heavy chains with additional 7×-His tags. The PG9 and PG16 constructs were subcloned into the mammalian expression vector pTT5 (NRC Biotechnology Research Institute). The scFv genes for PG9 and PG16 were constructed by inserting a 6×-His tag and stop codon after the second variable domain.

$VRC01_{scFv}$-PG16, $VRC01_{scFv}$-ZAGα3-Fc, and $VRC01_{scFv}$-E51 were constructed by combining the scFv gene from VRC01 IA with IgG heavy chain or Fc fusion constructs by PCR and enzymatic ligation techniques. All gene constructs were verified by complete sequencing.

Protein expression and purification. Proteins were expressed transiently in suspension HEK 293-6E cells (NRC Biotechnology Research Institute) using 25-kDa linear polyethylenimine (PEI) (Polysciences) for transfection as described previously (8). When expressing heterodimeric constructs, the heavy chain (HC) and light chain (LC) plasmids were mixed at a 1:1 ratio by mass. Cell culture supernatants were collected 6 days posttransfection. For Fc-containing constructs, supernatants were passed over protein A resin (Thermo Fisher Scientific), eluted using pH 3.0 citrate buffer, and then immediately neutralized. 7×-His-tagged Fabs and scFvs were purified using nickel-nitrilotriacetic acid (Ni-NTA) chromatography and eluted using 300 mM imidazole. All reagents tested in neutralization assays were purified by size exclusion chromatography using a Superdex 200 10/300 GL column.

In vitro neutralization assays. A previously described pseudovirus neutralization assay was used to evaluate the neutralization potencies of the reagents (21, 27). Neutralization assays were performed either by the Collaboration for AIDS Vaccine Discovery (CAVD) core neutralization facility (Table 1; see also Tables S1 and S2 in the supplemental material) or by our laboratory (Table 2) using the same protocol (21, 27). Briefly, pseudoviruses were generated by cotransfection of HEK 293T cells with an Env expression plasmid and a replication-defective backbone plasmid. Neutralization was determined by measuring the reduction in luciferase reporter gene expression in the presence of a potential inhibitor following a single round of pseudovirus infection in TZM-bl cells. Nonlinear regression analysis was used to calculate the concentrations at which half-maximal inhibition was observed (IC_{50}s).

Biosensor affinity measurements. A Biacore 2000 biosensor system (GE Healthcare) was used to evaluate the interactions of VRC01 reagents with gp120. Approximately 750 response units (RUs) of protein A was covalently immobilized on all flow cells of a CM5 biosensor chip using standard primary amine coupling chemistry (Biacore manual). VRC01 IA, $VRC01_{scFv}$-ZAGα3-Fc, and VRC01 IgG were then bound to three of the individual flow cells (~1,400 RUs each), with the fourth flow cell serving as a blank. A concentration series of gp120 from strain Q259.d2.17 (expressed in baculovirus-infected insect cells as described previously [6]) was injected at room temperature in 10 mM HEPES with 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20 at pH 7.4. Equilibrium dissociation constants (K_Ds) were determined from kinetic constants derived from sensorgram data using simultaneous fitting to the association and dissociation phases of the interaction.

RESULTS

In order to compare the neutralization potencies of various antibody forms, we produced IgG, IA, and Fab forms of the anti-HIV antibodies PG9, PG16, and VRC01 (42, 44) (Fig. 1). We also produced the PG9 scFv but were unable to express either the VRC01 or PG16 scFvs in isolation in sufficient quantities for testing. Proteins were expressed by transient transfection of mammalian cells, and purification was by protein A or Ni-NTA chromatography followed by size exclusion chromatography. The size exclusion chromatography profiles of the PG9 and PG16 IAs revealed large peaks corresponding to the expected products (Fig. 2), i.e., a single Fc unit with two scFvs, which we will subsequently refer to as an IA monomer. During size exclusion chromatography of the VRC01 IA, however, both aggregated and apparently dimeric forms were observed in addition to the expected product (Fig. 2). These larger forms were a significant fraction of the material eluted from protein A chromatography, in contrast to the PG9 and PG16 IA purifications, in which only trace amounts of larger oligomers were observed.

The proteins were evaluated in an Env-pseudotyped HIV-1 neutralization assay against a panel of 30 strains (Table 1; see also Table S1 in the supplemental material). As observed for other

TABLE 1 IC_{50}s for IgG, Fab, IA, and scFv forms of VRC01, PG9, and PG16[a]

		IC_{50} (nM) of[a]:														
		VRC01							PG9				PG16			
Virus	Clade	IgG	IgG-2A	Fab	IA	IA dimer[c]	$VRC01_{scFv}$-ZAGα3-Fc	$VRC01_{scFv}$-E51	IgG	Fab	IA	scFv	IgG	Fab	IA	$VRC01_{scFv}$-PG16
6535.3	B	6.9	8.2	40	>96	>96	>79	>49	4.1	170	1.7	3,000	285	1,800	1.9	48
QH0692.42	B	7.5	7.5	14.0	27	57	6.5	4.1	>670*	>2,000	>920	>1,700	>680*	>2,000	>930	14
SC422661.8	B	0.62	0.69	2.3	2.0	5.8	11	3.9	20	220	120.0	>3,500	120	850	200	2.1
PVO.4	B	4.4	4.6	10	20	49	23	23	80	500	>820	>1,700	89.5	260	>930	15
TRO.11	B	2.4	2.2	12	9.6	28	7.9	11	240	1,900	>920	>3,500	19.4	390	>930	5.4
AC10.0.29	B	5.8	5.1	16	19	64	32	36	0.53	5.4	>0.92	34	0.22	1.2	3.7	0.34
REJO4541.67	B	0.21	0.27	1.0	1.7	4.0	2.3	1.5	0.1	7.5	1.8	>104	0.11	26	45	0.15
TRJO4551.58	B	0.62	0.69	1.6	2.3	7.8	1.7	2.2	3.9	200	820	200	25.5	240	11	0.44
WITO4160.33	B	0.41	0.55	2.3	5.4	13	7.8	4.2	0.33	0.42	0.28	0.07	0.095	0.51	1.4	0.15
CAAN5342.A2	B	5.8	5.7	16	39	95	29	27	80	240	>46	>3,500	140	590	>930	24
THRO4156.18	B	36	35	70	77	95	75	39	270	1,500	>820	>3,500	85.5	2,000	>930	7.9
RHPA4259.7	B	0.27	0.27	1.0	0.96	2.8	0.79	1.1	180	1,500	>46	>1,700	3.4	53	>390	1.0
Du156.12	C	0.62	0.41	1.2	3.9	16	4.5	2.6	0.33	2.8	>0.92	2.4	0.16	0.67	4.9	0.29
Du172.17	C	>69*	>69	>620	>96	>96	>79	>49	3.3	9.30	9.0	220	0.41	2.6	12	2.3
Du422.1	C	>69*	>69	>620	>96	>96	>79	>49	2.9	7.1	>0.92	1,114	0.95	28	94	19
ZM197 M.PB7	C	4.3	3.2	6.9	21	84	16	17	6.7	13	18	43	8.1	73	33	5.6
ZM214 M.PL15	C	1.7	1.7	8.4	31	31	50	>49	1,500*	>2,000	>920	>1,700	>680*	>2,000	>930	>49
ZM233 M.PB6	C	5.3	12	220	>96	>96	>79	>49	0.23	0.20	0.28	0.31	0.034	0.081	0.88	0.05
ZM249 M.PL1	C	0.62	0.82	1.4	2.7	11	2.1	2.6	1.4	0.99	4.7	2.1	0.81	4.3	3.7	0.34
ZM53 M.PB12	C	4.6	4.4	15	22	72	18	27	0.4	3.9	8.2	56	0.25	1.1	3.2	0.34
ZM109F.PB4	C	1.5	1.5	7.2	6.7	22	2.9	3.8	1.0	260	19	1,200	134	>2,000	15	3.1
ZM135 M.PL10a	C	4.9	5.3	41	13	36	25	26	>6.7*	560	>46	>3,500	>680*	>2,000	>930	37
CAP45.2.00.G3	C	44	23	>620	>96	>96	>79	>49	0.020	0.12	0.18	0.035	0.027	0.061	9.0	0.05
CAP210.2.00.E8	C	>48*	>48	>620	>67	>67	>79	>49	3.1	6.2	18	490	0.47	14	51	2.8
Q23.17	A	0.41	0.55	4.3	2.3	6.8	3.1	1.9	0.067	<0.99	0.18	0.14	0.014	0.20	0.45	<0.024
Q842.d12	A	0.34	0.34	0.62	0.77	3.0	0.87	1.0	0.20	1.3	0.65	0.70	0.18	0.63	3.5	0.15
Q259.d2.17	A	0.69	0.34	110	41	62	>79	>49	1.5	0.99	1.8	45	0.068	19	3.4	1.6
3718.v3.c11	A	2.5	2.5	130	90	96	>79	>49	0.47	2.9	2.3	2.4	0.068	2.1	24	1.4
0330.v4.c3	A	0.41	0.27	1.2	1.8	5.6	2.1	2.4	0.13	0.97	0.46	0.70	0.14	0.69	2.8	0.24
3415.v1.c1	A	0.34	0.27	1	2.1	7.1	1.8	2.5	0.53	1.8	4.3	7.3	0.14	2.3	22	0.59
Geometric mean		1.8	1.7	8.8	10.5	24.5	10.7	11.0	1.9	13.3	6.5	42.0	1.2	12.7	18.9	1.4
MNR[b]			1.0	5.0	5.9	13.9	6.1	6.2		6.8	3.3	21.6		10.5	15.5	

[a] *, IC_{50} for this strain was excluded from the calculation of the geometric mean so that results could be compared with those for the IgG forms.

[b] MNR, molar neutralization ratio of the indicated reagent compared to results for the parental IgG.

[c] IC_{50}s given for the VRC01 IA dimer fraction were calculated using the monomer molecular weight. IC_{50}s (in μg/ml) are presented in Table S1 in the supplemental material.

TABLE 2 Effect of preincubation of VRC01 reagents on neutralization potency[a]

Pseudovirus	Reagent	IC_{50} (nM) of reagent at preincubation time		
		0 h	12 h	24 h
JR-FL	VRC01 IgG	0.37	0.40	0.31
	VRC01 IA	5.2	4.6	1.7
Du156	VRC01 IgG	0.67	0.60	0.47
	VRC01 IA	3.1	2.6	2.0

[a] The neutralization assay was modified by incubating the reagent in assay medium for the indicated times prior to addition of pseudovirus.

anti-HIV antibodies, the Fab forms were on average 5- to 10-fold less potent on a molar basis than the intact IgG (18). The 2A form of VRC01 IgG (translated from a single mRNA containing a picornavirus 2A peptide sequence [37] between the heavy and light chain genes) had potency equal to that of VRC01 IgG. However, the IA forms of VRC01, PG9, and PG16 were less potent than the corresponding IgGs; the potencies were reduced overall by 5.9-fold (VRC01), 3.3-fold (PG9), and 15.5-fold (PG16 IA, constructed as V_L followed by V_H). Two-tailed paired *t* tests demonstrated that these differences were significant (*P* values of 0.00045, 0.047, and 0.032, respectively). A PG16 IA constructed with V_H followed by V_L was also expressed and tested on a more limited number of strains; this reagent was also less potent than PG16 IgG (see Table S2 in the supplemental material). Although the dimeric fraction of the VRC01 IA was active in neutralization, it was 2.3-fold less potent on a mass basis (*P* value of 0.00012) than monomeric VRC01 IA (Table 1).

Potential reasons for the IAs to be less potent than their parent IgGs include the following: (i) a shorter span between the two antigen combining sites, (ii) reduced stability of IAs versus IgGs in the assay media, and/or (iii) reduced affinity of the scFv antigen binding sites compared to that of Fab binding sites. To evaluate whether the shorter arm span of the IAs diminished their activity, we expressed an scFv-containing reagent with a combining site separation more similar to that of IgG by inserting an immunoglobulin constant region-like domain, the α3 domain from Zn-α2 glycoprotein (ZAG), between the scFv and Fc components to create $VRC01_{scFv}$-ZAGα3-Fc (Fig. 1). The maximal separation between the V_H-V_L combining sites should be similar in $VRC01_{scFv}$-ZAGα3-Fc and VRC01 IgG. To permit even greater separation, the VRC01 scFv was also attached to the N terminus of the heavy chain of an unrelated IgG to create $VRC01_{scFv}$-E51. The CD4-induced (CD4i) antibody E51 was chosen for this construct because E51 IgG expresses well and is weakly neutralizing or non-neutralizing in the absence of CD4 (22), as observed for other CD4i antibodies (19). Neutralization assays using these reagents (Table 1) demonstrated an average IC_{50} similar to that with VRC01 IA, indicating that the arm span of $VRC01_{scFv}$-based reagents had little impact on their neutralization potencies.

We next tested whether differences in reagent stability (i.e., survival) under our neutralization assay conditions contributed to the differences we observed between IAs and IgGs by conducting assays with a 12- or 24-h preincubation in assay medium prior to adding pseudovirus. No trend toward diminished neutralization potency over time was observed (Table 2), demonstrating that differential stability in the assay medium did not account for differences in potency.

A weaker antigen-binding affinity of the scFv in an IA versus the Fab in an IgG was suggested by the less potent neutralization observed for PG9 scFv than for PG9 Fab (Table 1). The possibility of reduced antigen-binding affinity was directly tested by comparing the binding of gp120 to VRC01 IgG versus scFv-containing

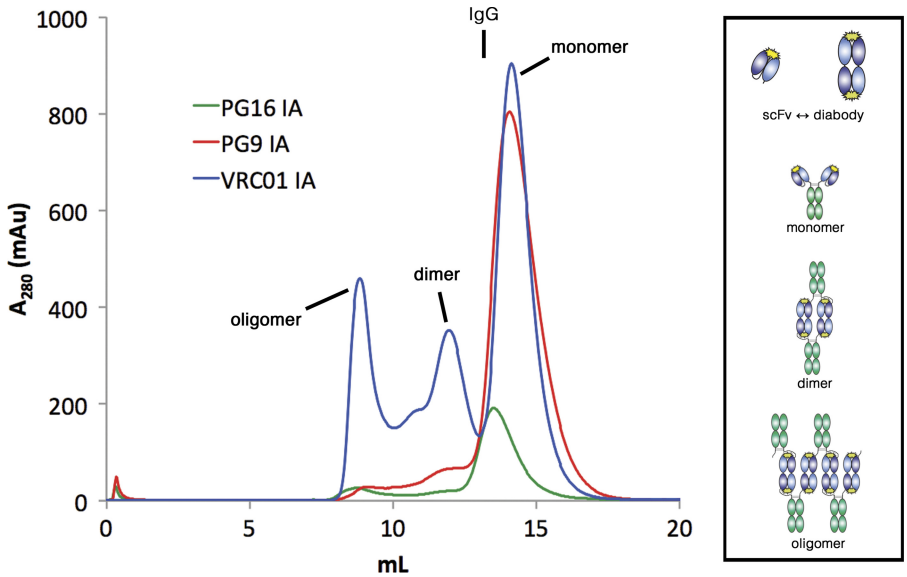

FIG 2 Size exclusion chromatography profiles of PG16, PG9, and VRC01 IAs. Protein A-purified IAs were injected over a Superdex 200 10/300 GL column. IgGs normally elute at ~13 ml (as indicated at the top of the figure), compared to the slightly smaller IA monomers, which elute at ~14.5 ml. (Data are shown for the V_L-V_H version of the PG16 IA; similar results were obtained for the V_H-V_L version.) Although the PG9 and PG16 IAs were predominantly monomeric, the VRC01 IA profile showed multimeric (presumably dimeric) and aggregate peaks in addition to the monomer. The tendency of scFv molecules to dimerize by 3-D domain swapping (shown schematically at the top in the box) may lead to the formation of dimeric and oligomeric forms of IAs (potential structures shown in the box).

forms of VRC01. Purified gp120 from strain Q259.d2.17 was injected over protein A-immobilized VRC01 IgG, VRC01 IA, or $VRC01_{scFv}$-ZAGα3-Fc in a surface plasmon resonance (SPR)-based binding assay (see Fig. S2 in the supplemental material). The equilibrium dissociation constant (K_D) derived for VRC01 IgG was 160 nM, compared with affinities of 590 nM and 570 nM for VRC01 IA and $VRC01_{scFv}$-ZAGα3-Fc, respectively. The approximately 4-fold-weaker affinity of the scFv-containing reagents was comparable to the 6-fold-weaker neutralization IC_{50} of VRC01 IA compared to that of VRC01 IgG.

To explore the potential for combined reagents to provide greater neutralization breadth, we expressed a modified form of PG16 in which a VRC01 scFv was attached to the N terminus of the PG16 light chain with a $(Gly_3\text{-}Ser)_6$ linker. The neutralization properties of $VRC01_{scFv}$-PG16 are shown in Table 1. Inspection of the measured IC_{50}s for strains that were resistant to either VRC01 or PG16 confirmed that both components in the combined reagent were active.

We assessed the potencies of each of the components in the bispecific $VRC01_{scFv}$-PG16 reagent by a modeling procedure using the IC_{50}s for VRC01 IgG and PG16 IgG as follows. Assume the IC_{50}s of the parent IgGs VRC01 and PG16 are v and p, respectively, for a given HIV-1 strain. Consider an idealized combined reagent in which both components functioned independently with no synergy or interference. In a very simplified picture of virus neutralization, reagent binding is equivalent to neutralization, and the IC_{50} can be approximated by a single binding event with the same equilibrium dissociation constant. Solving the equilibrium equations for 50% binding/neutralization, we found the modeled IC_{50} was $(\sqrt{v^2 + 6vp + p^2} - v - p)/2$. In the actual bispecific reagent, we anticipated the individual components would have reduced activity, i.e., $v_{reduced} = v_{eff} \times v$, where v_{eff} is >1, and $p_{reduced} = p_{eff} \times p$, where p_{eff} is >1. Assuming that v_{eff} and p_{eff} are constant across different strains, we solved for best-fit values of these parameters that minimized $\Sigma_{strains}(\log IC_{50\ observed} - \log IC_{50\ modeled})^2$. A fit assuming only one active antibody did not fit the data as well as a fit assuming that both components were active (see Fig. S3 in the supplemental material).

Using our neutralization data for $VRC01_{scFv}$-PG16 (Table 1), we derived values of 3.16 and 3.22 for v_{eff} and p_{eff}; thus, the VRC01 scFv component possessed about 1/3 of the potency of VRC01 IgG, and the PG16 IgG portion possessed about 1/3 of the potency of unmodified PG16 IgG. For any given strain, the combined reagent was nearly always weaker than the stronger parent IgG. However, the combined reagent was superior on a mass basis to the weaker parent for 27 out of 30 strains (Fig. 3, bottom panel). In addition, the combined reagent neutralized more strains than either parent; e.g., using an IC_{50} of <5.0 μg/ml as the cutoff for neutralization, $VRC01_{scFv}$-PG16 neutralized 90% of the strains we tested, while VRC01 IgG neutralized 83% and PG16 IgG neutralized 70% (Table 1 and Fig. 3, top panel). However, this depends on the neutralization threshold chosen. For IC_{50}s of <1.0 μg/ml, the combined reagent neutralized 67% of strains, while VRC01 and PG16 neutralized 77% and 60%, respectively.

DISCUSSION

Provision of prophylactic or therapeutic antibodies by direct injection or gene therapy permits consideration of a wide range of potential reagents, building on initial discoveries of anti-HIV IgGs. Variations fall into three major categories: (i) those affecting the antigen binding site, (ii) choice of the overall architecture (e.g., IA versus IgG), and (iii) modulations of effector function. A thorough evaluation of the efficacies of the full range of potential reagents is a large task. A variety of selection strategies is available to screen variants in category i, i.e., natural antibody repertoires and antigen binding site libraries. However, full exploration of the alternatives in categories ii and iii is limited by the need to produce purified, testable quantities of reagents and the screening limitations of evaluating effector function in complex cell- or animal-based assays. The present studies are intended to provide insight into the potential effects of architecture on reagent potency.

The size of a delivered reagent must be considered for gene therapy efforts involving AAV. In general, constructs approaching the size limit for packaging AAV suffer reduced expression levels (7). Although the minimum serum or genital tract anti-HIV IgG concentration necessary for protection is not known, a rough estimate of 100 times the reagent's IC_{50} has been suggested (30). For broad strain coverage with reagents such as VRC01, PG9, or PG16, this implies desired concentrations in the tens to hundreds of μg/ml. Early efforts using an AAV vector with separate promoters for heavy and light chains directing expression of anti-HIV IgG b12 yielded serum concentrations of only $\sim$5 μg/ml (20). Alternative AAV/IgG constructs have permitted high-level IgG expression in other cases. For example, use of a single promoter with a 2A self-processing peptide inserted between the antibody heavy and light chains permitted $\sim$1-mg/ml IgG levels to be achieved with AAV-transduced liver expression (9). Nevertheless, the smaller size of IA constructs versus conventional IgG is attractive for gene therapy approaches in which vector capacity is severely limited—in particular, for self-complementary AAV vectors, which are more efficient at transduction than AAV vectors with a single-stranded genome (25).

To more fully understand the potential trade-offs in vector and construct design, we compared the neutralization activities of IA versus IgG versions of three broadly neutralizing antibodies: PG9, PG16, and VRC01 (42, 44). We found that the PG9, PG16, and VRC01 IAs were severalfold less potent than their IgG forms. A reduced affinity of the scFv antigen-binding site is a likely contributing factor to this difference. Although some scFvs have affinities equivalent to those of the related Fabs, it was noted in early studies that scFvs can exhibit up to 10-fold weaker binding (4). The weaker binding and neutralization by the VRC01 scFv-containing reagents is likely due to suboptimal geometry of the antigen binding site and/or steric interference by the Gly-Ser linker joining the VRC01 V_H and V_L domains. Steric interference from the scFv linker is consistent with the VRC01 Fab-gp120 crystal structure (45), in which the N terminus of the Fab light chain is $\sim$8 Å from the gp120 backbone. This relatively close distance suggests that the Gly-Ser linker extending from the V_L domain N terminus could sterically interfere with antigen binding. It is possible that the scFvs in the reagents we tested were suboptimal; different designs might yield scFvs with affinities matching those of the corresponding Fabs. Thus, to achieve maximal efficacy, it will be necessary to explore different architectures (V_H-V_L versus V_L-V_H) and different linker lengths for each scFv used in IAs.

A potential complication of scFv reagents is their tendency to dimerize or multimerize by three-dimensional (3-D) domain swapping (3). The extent of dimerization of scFvs is variable, depending on linker length, antibody sequence, concentration, buffer conditions, and the presence or absence of antigen (1). The

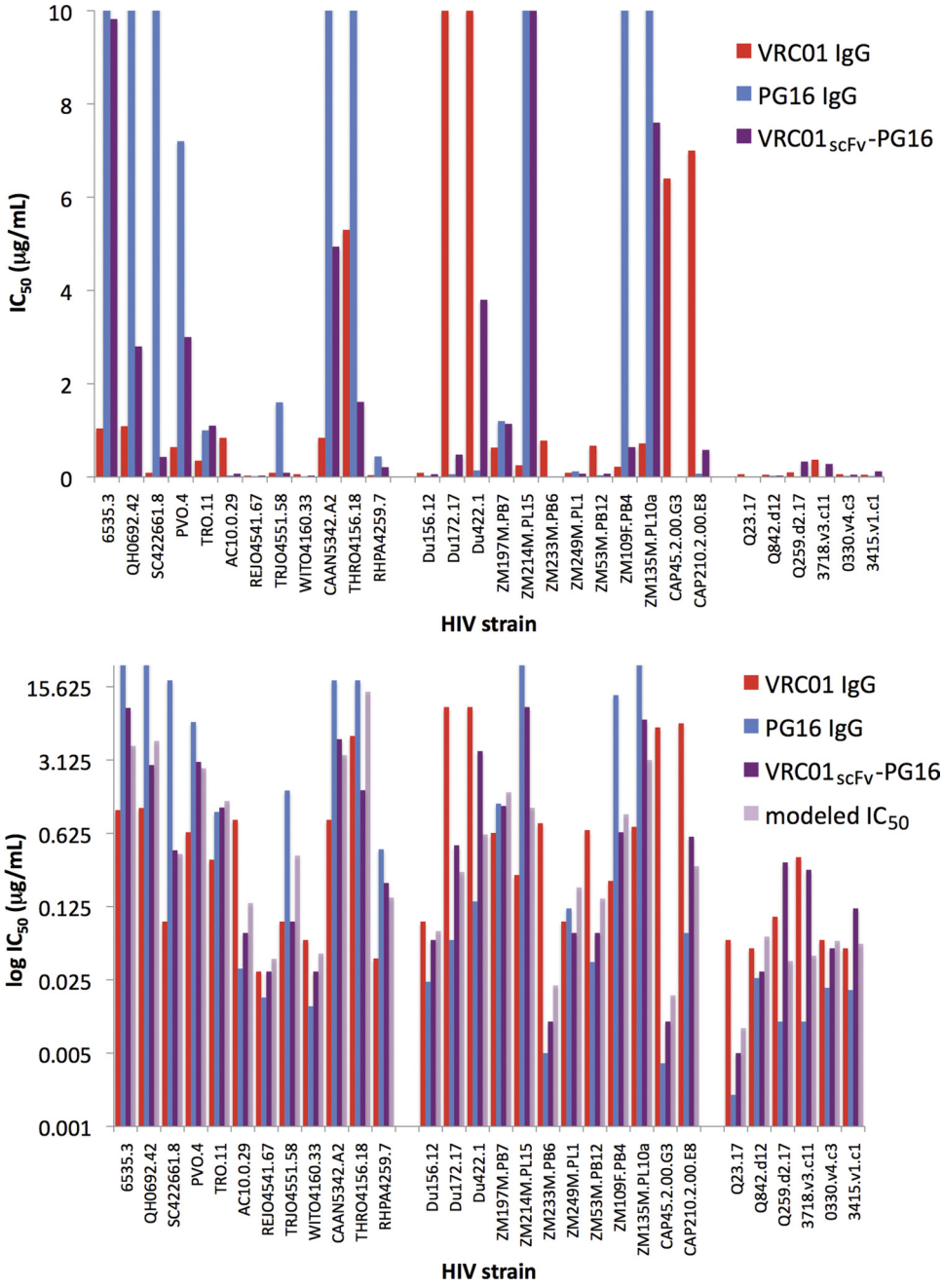

FIG 3 Histograms displaying IC_{50}s (μg/ml) of VRC01 IgG (red), PG16 IgG (blue), and $VRC01_{scFv}$-PG16 (purple) (top; linear scale) or VRC01 IgG (red), PG16 IgG (blue), $VRC01_{scFv}$-PG16 (purple), and the modeled IC_{50}s of $VRC01_{scFv}$-PG16 (light purple) (bottom; logarithmic scale).

potential for IAs to form dimers or other oligomers as observed for VRC01 IA is a special concern for reagents that will be delivered by gene therapy, where it is not possible to remove higher-order products once they are secreted from transduced cells. Multimeric forms of IAs may be less potent and potentially more immunogenic than the monomeric molecule; this may add to the immunogenicity of the artificial linker of IAs. AAV-mediated expression of IAs in rhesus macaques led to various levels of IA-specific antibody response, which appeared to be correlated with reduced efficacy against viral challenge (15). The best means of addressing this possible complication is careful biophysical characterization of proposed gene therapy protein products.

In the recent rhesus macaque SIV challenge experiment, delivery of IAs was found to be superior to either scFv or whole IgG with respect to the serum concentrations that could be achieved (15). Although the IAs used in the challenge experiments exhibited neutralization IC_{50}s well below 1.0 μg/ml, the Fabs from which they were derived had IC_{50}s that were 3-fold more potent on a mass basis (16). (The IC_{50}s of the corresponding IgGs have not been reported). On a molar basis, the IAs were thus about

1.5-fold less potent than the Fab forms. This ratio is very similar to the average 1.2-fold- and 1.5-fold-weaker molar neutralization we observed for the VRC01 and PG16 IAs versus the corresponding Fabs (Table 1). In the rhesus challenge study, the IA potencies (IC_{50}s of 0.01 to 0.02 μg/ml against the SIVmac316 challenge strain) were sufficient to provide protection. However, since anti-HIV IgGs are generally 6- to 30-fold more potent than their corresponding Fabs on a molar basis (18), the IAs evaluated here represent a significant trade-off necessitated by the lower serum IgG concentrations achievable (15) with currently available AAV technology.

While VRC01, PG9, and PG16 have very broad activities, each of these antibodies fails to neutralize 9 to 27% of HIV-1 strains (using a cutoff IC_{50} of 50 μg/ml; at 1 μg/ml, 28 to 49% of strains are not neutralized) (42, 44). For passive immunization or gene therapy applications, addressing the incomplete strain coverage requires delivering either multiple antibodies or a single reagent combining two or more activities. Here we investigated the feasibility of one such bispecific reagent, in which a VRC01 scFv was attached to PG16 IgG ($VRC01_{scFv}$-PG16). *In vitro* neutralization assays against a panel of HIV-1 strains demonstrated both VRC01 and PG16 activities. Although both potencies were reduced compared to those of the parental IgGs, $VRC01_{scFv}$-PG16 showed greater breadth, suggesting the potential for a bispecific reagent to provide complete or near-complete protection against HIV-1. Further development of such a reagent is possible, for example, given that the VRC01 scFv component of $VRC01_{scFv}$-PG16 had a reduced activity similar to that observed for the VRC01 IA, improvement of the scFv portion could be attempted. The weaker activity of the PG16 component may result from steric factors from the scFv attached to the N terminus of the PG16 light chain. Switching the VRC01 scFv to the N terminus of the PG16 heavy chain did not improve PG16 activity (see Table S3 in the supplemental material). Changing the size or structure of the linker or switching to the C terminus of the heavy chain may permit greater PG16 activity. Although the reduced potencies of the individual components of a bispecific reagent might increase the risk that resistance to these neutralizing activities could develop, this concern is arguably secondary to providing breadth of coverage in the context of infection prophylaxis (versus treatment) since the goal of infection prophylaxis is to neutralize a small viral inoculum rather than to control an established infection.

The design of gene therapy reagents for HIV-1 prophylaxis potentially involves a variety of trade-offs, including breadth of reagent, potency, expression level, and minimization of potential for immunogenicity and other side effects. The newly discovered anti-HIV antibodies (42, 44) demonstrate that breadth and great potency can be achieved simultaneously. Our results suggest that careful optimization of reagent architecture and full biophysical characterization of the oligomeric states of potential protein reagents are important for fully exploiting the potential offered by genetic approaches to providing HIV-1 immunity. Direct conversion of IgGs to IAs will often entail a loss of potency due to weaker binding of the scFv compared to the Fab binding site and/or domain swapping to create scFv multimers, which can perhaps be avoided by screening many scFv designs. Whether this loss of potency is acceptable depends on relative *in vivo* serum levels of AAV-expressed IAs and IgGs. For prophylaxis against a wide variety of circulating HIV-1 strains, a delivered reagent will face strains where its activity is far from maximal. In this situation, optimization of the reagent will be critical to provide robust protection.

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